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Determination of relative protein degradation activity at different life stages in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Rainbow trout were reared from 5 g to ~400 g on a diet formulated to supply the required protein from either fishmeal or plant proteins. The fish were sampled at every weight doubling and liver and muscle samples were obtained. From these tissue samples RNA and protein were isolated and analyzed for the expression of a number of muscle regulatory and protein degradation genes and enzymatic activity for proteins involved in the caspase, calpain, and ubiquitin-proteasome pathways for protein proteolysis. Only MyoD2 showed significant differences in expression between the two diets, while no significant changes over the course of the experiment were determined for MyoD2 or the other muscle factors. For the degradation genes significant changes in expression were determined for calpain1 and calpastatin. Calpastatin also showed a significant increase in expression over the course of the experiment in the muscle of fish fed a fishmeal diet and significant decrease in expression in the liver of fish fed the fishmeal based diet. Differences in proteasome enzyme activity were found between diets in the liver and muscle of fish and for caspase-3 activity in muscle. Significant changes in activity over the course of the experiment were noted for proteasome and calpain activity in the liver and muscle. These findings suggest that diets replacing fishmeal with plant material can have some effects on protein turnover in muscle and that some degradation pathways are differentially regulated during the growth of rainbow trout.

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1. Introduction

Freshwater aquaculture faces many constraints that limit its potential to expand. With reductions in available water and the rising costs of feeds, aquaculture producers are looking for ways to reduce costs and increase production. The use of genetic selection programs for improvement of valuable traits has proven to be an effective and economical method for increasing production (Harris and Newman, 1994). For aquaculture reared fish the fillet is the main product sold to consumers. Rather than increasing growth strictly by measuring weight gain as a trait, it would be of greater benefit to select for a production trait such as efficient muscle accretion and/or maintenance of protein turnover. The rate of fish growth varies dramatically at different life stages, especially from 5 g to around 300 (Ferreira and Russ, 1994; Hutchings and Jones, 1998). Regulation of protein turnover in muscle most likely plays a significant role in the variation of growth rate at a particular size or age. Physiological studies involving fish muscle development include analysis and expression of muscle differentiation and developmental factors, fiber and microtubular development, hypoplasia and hyperplastic growth, nutrient partitioning, and transgenically modified growth (Rescan, 2005; Johnston et al., 1999; Chauvigne et al., 2005; Johansen and Overturf, 2006; Devlin et al., 2001). Most of the research has been done with salmonids but a number of other species have also been the focus for muscle development studies (Rowlerson et al., 1995; Veggetti et al., 1993). Muscle growth in salmonids, and other vertebrates, is believed to be due to the synergistic responses of both muscle synthesis and degradation. While increased synthesis is a means to increase muscle accretion, it is hypothesized that management of muscle degradation is the checkpoint in directing the regulation of protein turnover and muscle deposition in the animal. Protein degradation and cellular turnover occurs mainly through the action of four distinct pathways, 1) ubiquitin targeted digestion of protein by the proteasome, 2) transport to lysosomes and digestion through the use of cathepsin, 3) apoptosis and digestion following the caspase cascade, and finally 4) digestion through the action of nonlysosomal intracellular calcium dependent calpains.

Research studies have looked at protein turnover and the activities of different degradation factors in rainbow trout during spawning, and at distinct phases of growth (Salem et al., 2005a, 2006a,b. Unlike in terrestrial mammals where protein turnover is carried out by the proteasomal digestion of ubiquitin targeted proteins, these studies indicated that fish appear to rely more

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Table 1
Composition of diets fed

Ingredient (g/100 g)	Diet 1 fish meal diet	Diet 2 plant meal diet
Fish meal, menhaden	47.68	
Barley meal, merlin	32.30	20.45
Wheat flour	9.70	
Wheat gluten		7.04
Corn gluten		34.57
Soybean meal		18.96
Fish oil	9.30	13.44
Vitamin premix	0.40	0.40
Trace mineral	0.10	0.10
Lysine-HCl		1.47
Taurine		0.50
Choline Cl	0.50	0.50
Dical		2.55
Stay-C	0.02	0.02
*Analyzed composition**		
Protein (%)	44.5	46.4
Fat (%)	19.2	13.6
Energy (cal/g)	5039.7	5233.1
dry matter (%)	90.98	96.33

*As-fed basis.

heavily on alternative pathways. Studies evaluating protein turnover in salmonids have found evidence of proteasome activity but have determined that it is probably not functioning as the main method for muscle degradation (Dobly et al., 2004; Martin et al., 2002; Kolditz et al., 2008; Seiliez et al., 2008). Cathespin and caspase gene expression and activity has been shown to be upregulated in deteriorating muscle of fertile spawning fish (Salem et al., 2006a,b). Cathespin was also found expressed at higher levels in the liver of short termed starved trout (Martin et al., 2001). Certain calpain isoforms have been shown to target and proteolyze specific muscle proteins in terrestrial vertebrates (Huang and Forsberg, 1998). And more recent findings by Salem et al. (2004, 2005a,b) have shown that calpain and some of its regulatory genes are active during muscle wasting and during the evaluation of fillet firmness in rainbow trout.

The dependence on fishmeal in salmonid diets is another economical and practical concern facing commercial aquaculture producers. If the growth of global aquaculture production continues as predicted over the next 20 years, then there will be insufficient fishmeal available to supply the protein necessary for commercial formulated feeds (Naylor et al., 2000). Therefore, a great amount of research has been done in developing salmonid diets that are formulated to replace a significant portion of the fishmeal protein currently used in aquaculture diets with more sustainable sources, notably plant proteins. In current feeds utilizing plant protein in lieu of fishmeal, and formulated to contain equal levels of protein, energy and other nutrient components, fish growth is still reduced by 10% or more (Barrows et al., 2008; Pierce et al., 2008). This reduction in growth might be due to a combination of a lack of micronutrients, improper amino acid balance, missing growth factors that have not yet been identified, the incorporation of anti-nutritional factors or an alternative energy partitioning of the nutrients from the diets. Although the factor/factors limiting complete removal of fish meal from trout feeds has not been defined, plant-based diets for rainbow trout may influence the expression or activity of protein degradation pathways. In related research completed with rainbow trout, a genotype by diet interaction for growth on plant-based feeds was found (Pierce et al., 2008).

Therefore, the intent of this research study was two-fold: 1) to evaluate the expression and enzymatic activity of three specific degradation pathways during the critical early growing stages of rainbow trout, and 2) to determine if the use of plant protein has an effect, negative or positive, on these degradation pathways.

Table 2
Gene name and accession number and primer and probe sequence for the genes analyzed by RT-PCR

Gene	Genebank accession no.	Primer/probe sequence (listed 5'-3')
β-actin	AF254414	BactinF: CCCTCTTCCAGCCCTCCTT BactinR: AGTTGTAGGTGGTCTCGTGGATA BactinMGB: 6FAM-CCGCAAGACTCCATACCGA-NFQ
calpastatinL	CA045868	CalpastatF: GCTCCAGCTGTCCATGCT CalpastatR: GCATCCAAGGCAAAGTCATCTGA CalpastatMGB: 6FAM-CCCCAGCTCCTCCC-NFQ
calpain1	AY573919	Calp1F: AGGCGCACGGGAACAG Calp1F: AGGCGCACGGGAACAG Calp1MGB: 6FAM-CCGCAGCCTGTTTGAG-NFQ
caspase-3	TIGR TC106471	Casp3F: AGCTGCTTTACACTGTGTCTCAA Casp3R: GCTCAGCATCACACACACAAAG Casp3MGB: 6FAM-ACAGCCAGTCAGCCTC-NFQ
Fructose 1,6-bisphosphatase	AY113693	FR16bPaseF: CGTTATGTCGGCTCCATGGT FR16bPaseR: TGCCTCCGTACACCAGAGT FR16bPase-MGB: 6FAM-CCTGTGCACATCAGCC-NFQ
Proteasome 20 delta	AF115539	ProtDF: GAGGGTCAGGATCCACCTATATCTA ProtDR: GCGAAGACACTGGTCCTTTGT ProtDMGB: 6FAM-ACTCCAACTACAAACCC-NFQ
TMyoD2	Z46924	MyoD2F: GCCGTCACCGACCAACT MyoD2R: CACTGTGTTCATAGCACTTGGTAGA Myod2MGB: 6FAM-CCGTCCCATGACCCC-NFQ
Tmyostatin1	AF273035	Myostatin1F: CCGCCCTTCACATATGCCAA Myostatin1R: CAGAACCTGCGTCAGATGCA MyostatinMGB: 6FAM-CATATTACATTTGGGATTCAA-NFQ
Tmyogenin	Z46912	Myogenin-382F: CATGGACCGGCGGAAAG Myogenin-400R: GGCCTCGAATGCCTCGT Myogenin-377MGB: 6FAM-CTTCTTCAGCCTCCTCTT-NFQ

2. Materials and methods

2.1. Fish rearing conditions and experimental design

All fish rearing was carried out at the University of Idaho's Hagerman Fish Culture Experiment Station. Rainbow trout (*Oncorhynchus mykiss*, Salmonidae) used in this study consisted of a background mixture of Housecreek, Kamloops, and Oregon strains, produced from a single

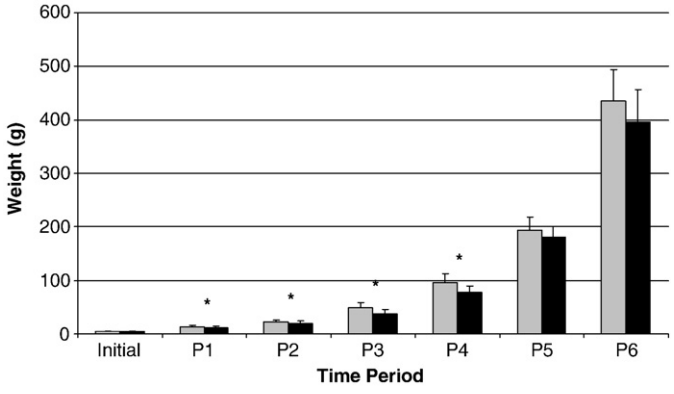

Fig. 1. Weight for fish fed either the plant based (black) or fish meal (gray) based diet at each sample time period. (* shows a significant difference in weight between the two diets $P<0.05$).

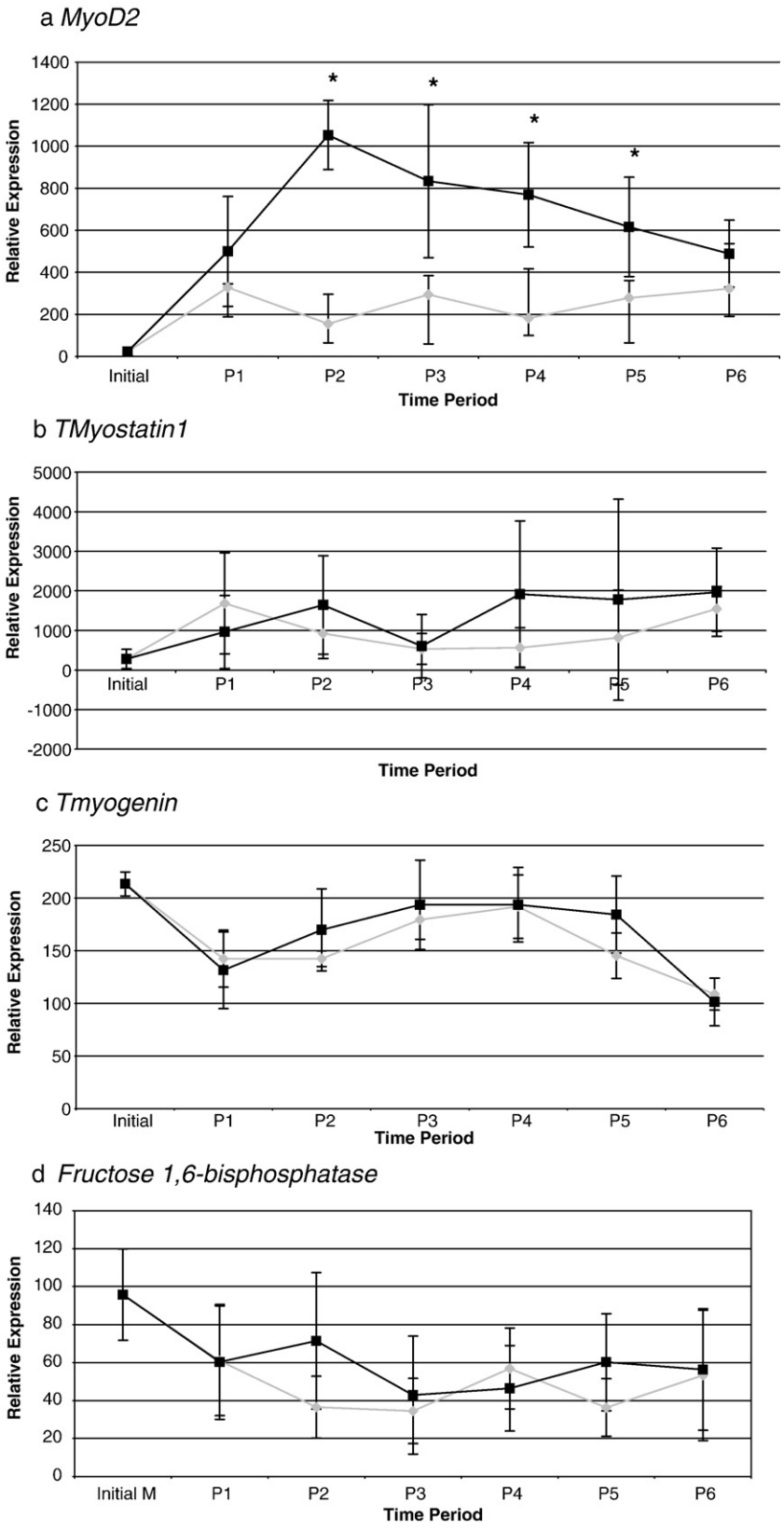

Fig. 2. a-d. Expression of muscle growth regulatory genes in fish fed either a plant based (black) or fish meal (gray) based diet analyzed at weight doublings from 5 g to 400 g. (* shows a significant difference in expression level between the two diets $P<0.05$).

paired mating. The experiment was carried out using 150 fish/tank in 575 L tanks with a water flow of approximately 30 L/min in constant temperature water of 15 °C, photoperiod was maintained at a constant 14 h/day. Fish were separated into two groups with one group reared on a standard fish meal based protein diet (Diet 1) and the other group reared on plant meal based protein diet (Diet 2) (Table 1). At the start of the

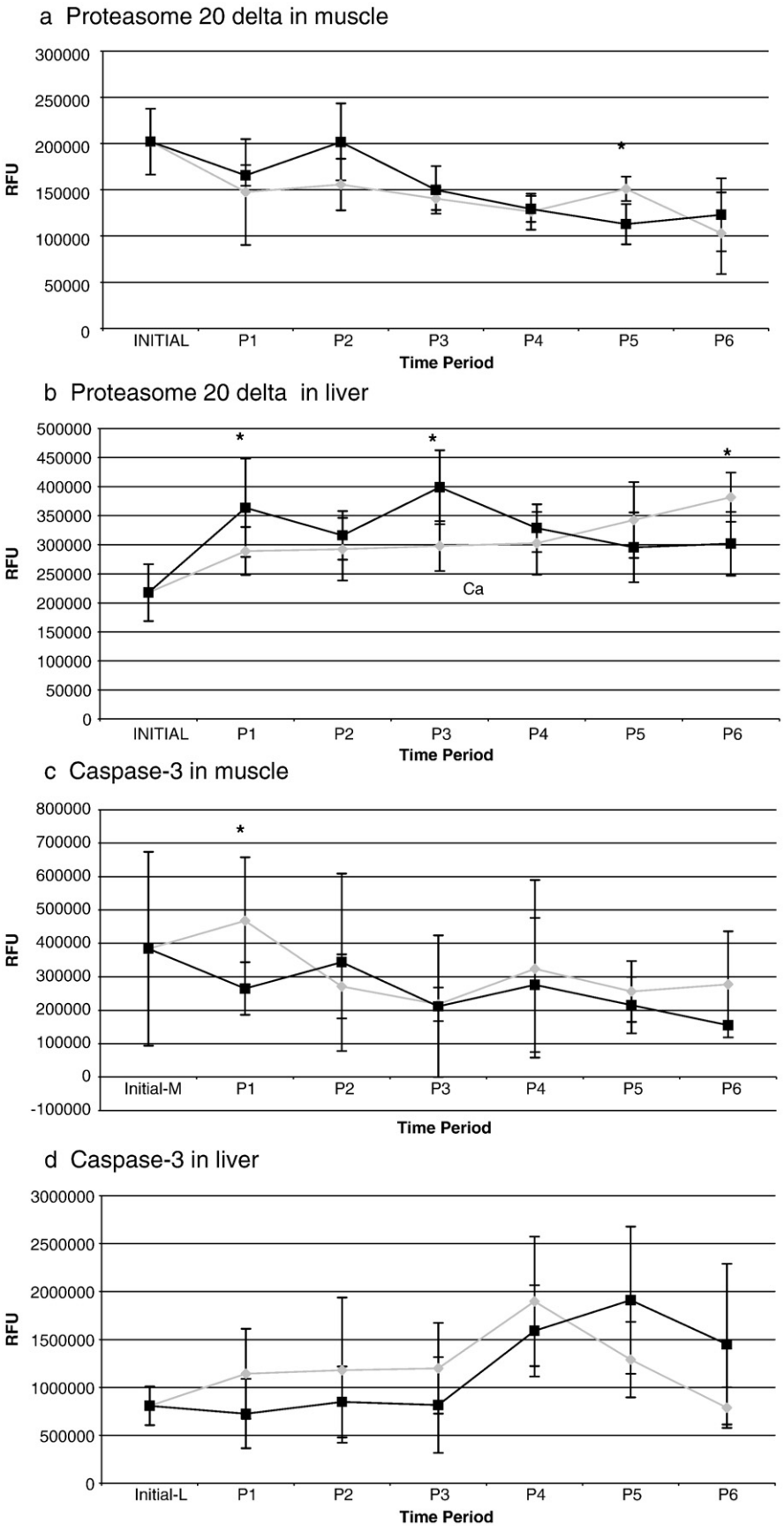

Fig. 3. a–h. Expression of protein degradation regulatory genes in the muscle and liver of fish fed either a plant based (black) or fish meal (gray) based diet analyzed at weight doublings from 5 g to 400 g. (* shows a significant difference in expression level between the two diets $P<0.05$).

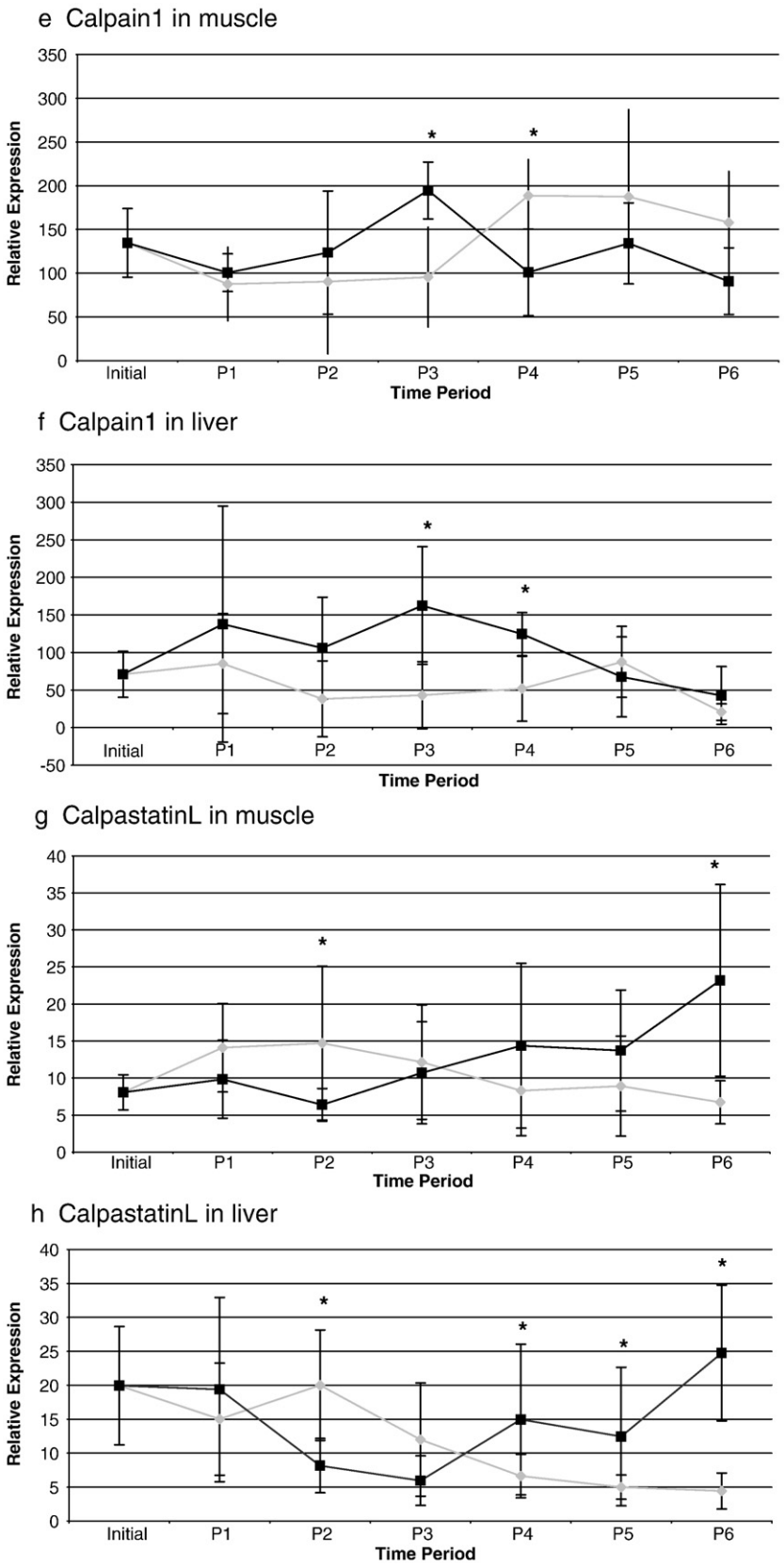

Fig. 3 (*continued*).

study the fish averaged 4.3 g. Initial tissue samples were taken from 10 fish prior to being separated into different dietary groups and then from 10 fish per dietary group for each diet at every weight doubling for 6 sampling time points. The sampling times correspond with P1 = 20 days, at P2 = 39 days, P3 = 55 days, P4 = 76 days, P5 = 110 days and P6 = 167 days from the start of the experiment. Fish on each diet were fed to satiation 3 times a day for 6 days a week throughout the duration of the study. Feed particle size was changed accordingly with fish growth.

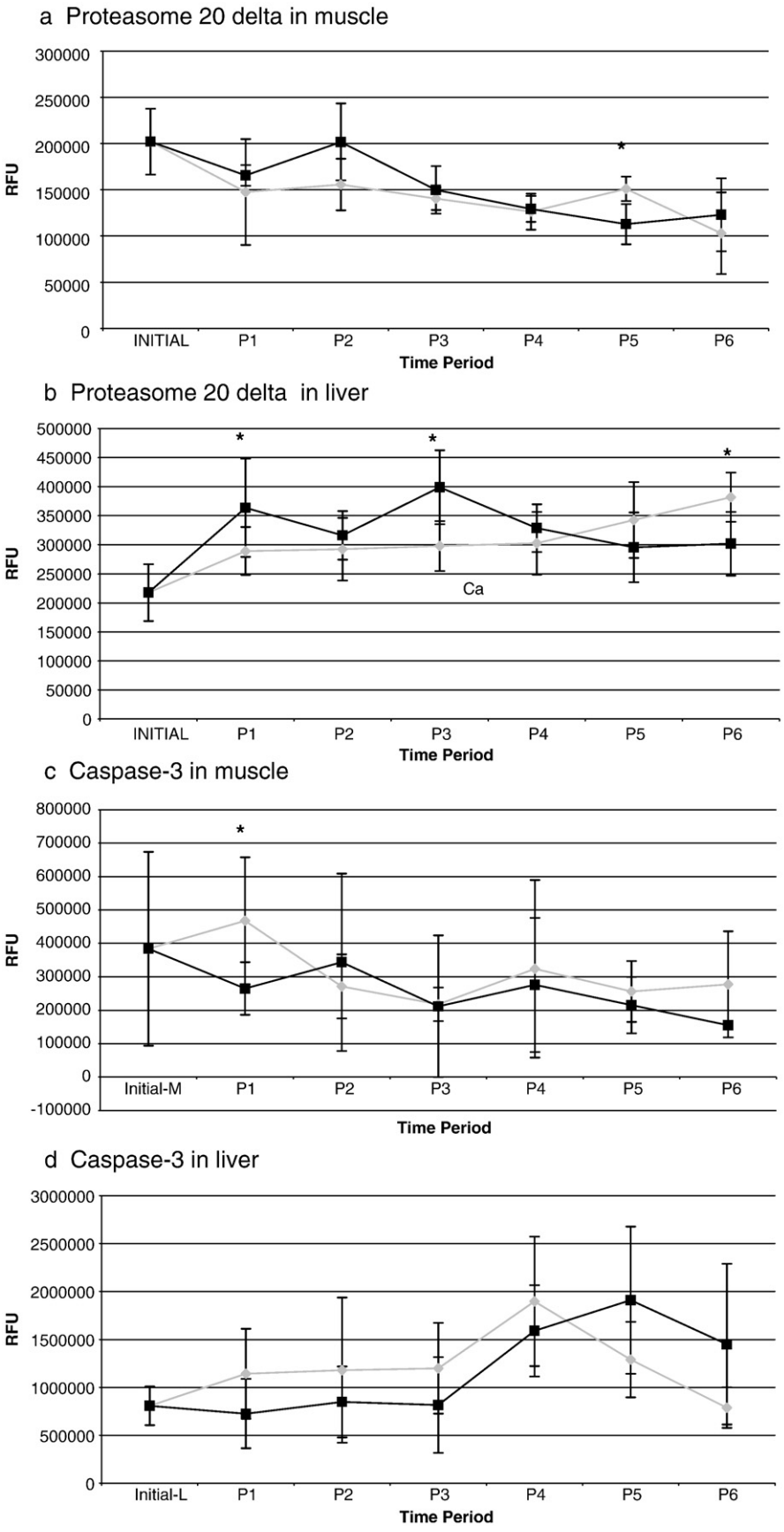

Fig. 4. a–f. Enzymatic activity of protein degradation enzymes in the muscle and liver of fish fed either a plant based (black) or fish meal (gray) based diet analyzed at nass doublings from 5 g to 400 g. (* shows a significant difference in expression level between the two diets $P<0.05$).

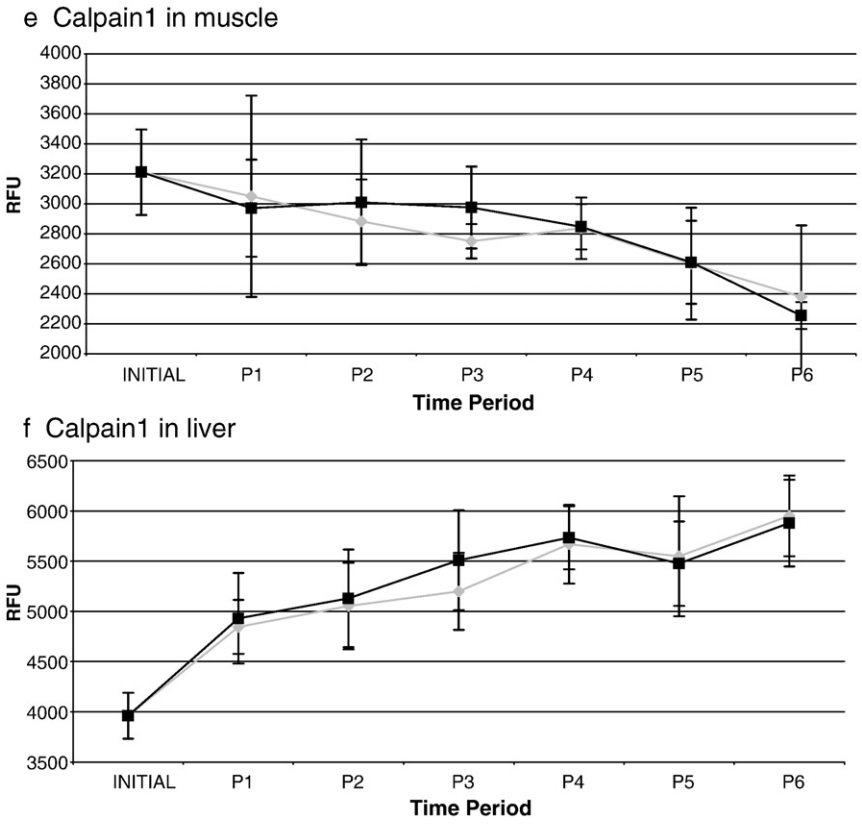

Fig. 4 (*continued*).

2.2. Sample collection and total RNA and protein isolation

At every time point when the average weight of the fish, obtained by subsampling, had increased twofold, 10 fish from each dietary group were removed from their respective tanks and liver and muscle tissue was collected. Samples were taken 14 h post feeding. RNA and protein was isolated from white muscle and liver at all time points (7) with an $n = 10$ for each diet at every time point. All total RNA isolations were carried out using TRIzol according to the manufacturer's protocol (Invitrogen, Rockville, MD, USA). Tissues were homogenized by adding a 5 mm stainless steel bead to the RNA-TRIzol mix and then shaking twice for 2 min at 40 Hz using a Qiagen MM301 shaker (Valencia, CA, USA). Total protein was isolated by placing approximately 80 mg of tissue into a 2 mL tube containing 500 μL of protein extraction buffer (0.3 M HEPES, 140 mM KCl, 5 mM $MgCl_2$, and 1X of Halt Protease Inhibitor/100 mL (Pierce, Rockford, IL, USA). Tissue was homogenized by adding a 5 mm stainless steel bead and shaken twice for 1 min at 18 Hz. The tubes were then placed on ice for 20 min prior to centrifugation for 1 min at 10,000 g and the supernatant was transferred to a fresh tube. RNA concentrations were determined spectrophotometrically using an Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany). Protein concentrations were determined using the Pierce BCA protein assay (Rockford, IL, USA) according to the manufacturer's protocol, and reading the plates on a Victor 3 multilabel plate reader (Perkin Elmer, Fremont, CA, USA).

2.3. Quantitative real-time RT-PCR

To detect the level of gene expression at each time point, real-time quantitative RT-PCR was carried out using an ABI Prism 7900HT Sequence Detection System and the TaqMan One-Step RT-PCR Master Mix Reagents kit from ABI, according to the protocol provided by ABI (Foster City, CA, USA). The final concentration of each reaction was: Master Mix, 1x (contains AmpliTaq Gold enzyme, dNTPs including dUTP, a passive reference, and buffer components); MultiScribe reverse transcriptase, 0.25 U/μL; RNase inhibitor mix, 0.4 U/μL; forward primer 600 nM; reverse primer 600 nM; probe, 250 nM; total RNA, 75 ng with water added for a total reaction volume of 15 μL. Probes and primer sequences are listed in Table 2. Cycling conditions for genes tested were as follows: 30 min at 48 °C, 10 min at 95 °C, then 40 cycles of PCR consisting of 15 s at 95 °C followed by 1 min at 60 °C. For each gene, assays were run in duplicate on RNA samples isolated from individual fish.

Relative copy number for the expression of each gene tested was determined by serially diluting a random experimental sample 10-fold and including this in each set of real-time assays run. In addition, as a cellular mRNA control, β-actin levels were determined for each sample and used in the normalization of specific expression data (Kreuzer et al., 1999). The data are reported as a ratio of relative mRNA copy number of each gene to absolute mRNA copy number of B-actin, multiplied by a constant for ease of interpretation and expressed as means ± standard errors.

2.4. 20 S proteasome activity assay

Evaluation of proteasome activity for protein degradation was performed using the BIOMOL QuantiZyme Assay System 20 S Proteasome Assay Kit for Drug Discovery (BIOMOL International, LP, Plymouth Meeting, PA, USA) which is designed to measure the chymotrypsin-like protease activity of the 20 S proteasome. Briefly, 80 μL of isolated protein at a concentration of 1.63 μg/μL, 10 μL of 10X assay buffer and 10 μL of substrate (Suc-LLVY-AMC fluorogenic peptide) are combined in a single well of a 96 well plate and incubated at 37 °C for 1 h. Activity was measured by determining cleavage of the AMC fluorophore by measuring emission at 460 nm (excitation: 360 nm). All enzymatic assays were measured in 96-well plates using a Victor 3 plate reader (Perkin Elmer, Waltham, MA, USA); all samples were run in triplicate.

2.5. Calpain activity assay

Calpain activity was measured using the BioVision Calpain Activity Assay Kit (BioVision Research Products, Mountain View, CA, USA). This assay measures calpain activity by detecting cleavage of a calpain substrate (Ac-LLY-AFC) which is then measured at 505 nm (excitation: 400 nm). Briefly, 85 μL of isolated protein at a concentration of 1.63 μg/μL, 10 μL of reaction buffer, and 5 μL of substrate were combined for each sample. Reaction plates were incubated for 1 h at 37 °C prior to reading.

2.6. Caspase-3 activity assay

Caspase-3 activity was measured using Sigma's Caspase 3 Fluorometric Assay Kit (Sigma-Aldrich, St. Louis, MO, USA). Caspase 3 is an effector caspase and can process caspases 2, 6, 7, and 9 proenzymes and specifically cleave most caspase related substances. This assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent7-amino-4-methylcoumarin (AMC) moiety. Reactions included 50 μL of protein at a concentration of 1.63 μg/μL and 50 μL of 2X extraction buffer/DTT mix. The reaction plates were incubated for 2 h at 37 °C prior to sample analysis.

2.7. Data analysis

Specific growth rate was calculated as equal to 100*(ln final mass of fish–ln initial mass of fish)/experiment days. The expression data is reported as a ratio of the relative mRNA copy number of each gene to the relative copy number of B-actin, multiplied by a constant for ease of interpretation, and expressed as means+standard errors. Microsoft excel was used to produce graphical representations of the data. T-test and linear regression analysis of the data was performed using SYSTAT ver. 9 (SPSS, Chicago, IL, USA).

3. Results

3.1. Growth data for fish on either the fish meal or plant meal based protein feed

For the first four sample periods after the fish were fed their respective diets, P1-P4, there was a significant weight difference between the fish on the different diets with the fish fed the plant based diet (Diet 2) weighing significantly less (Fig. 1). For the final two time periods the fish on the fish meal diet averaged heavier, 7% or 14 g heavier on time period 5 (day 110) and 9% or 40 g heavier on time period 6 (day 167), but the differences were not significant to the level of $P>0.05$, 0.09 and 0.07 respectively. Specific growth rates for the different diets over the course of the experiment were 2.71 for the plant meal based diet and 2.77 for the fish meal based diet.

3.2. Expression of muscle related genes

RNA isolated from the muscle of sampled fish was analyzed for the expression of genes which had previously been determined to correlate with changes in muscle development and growth related to protein accretion in rainbow trout or were believed to act in accordance with metabolic and growth changes (Johansen and Overturf, 2006; Johansen et al., 2006; Langley et al., 2002; Panserat et al., 2001). These tested genes included *TMyoD2*, *Tmyostatin1*, *Tmyogenin*, and *fructose 1,6-bisphosphatase* (*FBP*). For the gene *TMyoD2*, expression was found to be significantly different between the two diets during the time periods of P2 through P5 (Fig. 2a). In fish fed the plant-based diet *TMyoD2* displayed a significant increase in expression from between the initial sampling period to P2, $R^2=0.99$ and $P<0.01$, this was then followed by a significant decrease from time period P2 to P5, $R^2=0.99$ and $P<0.003$. For fish sampled on the fishmeal containing diet, no significant changes for *TMyoD2* expression were determined for the sampling time periods P1 through P6. Expression of *Tmyostatin1* was not significantly different between fish fed either of the two diets (Fig. 2b). Expression of *Tmyogenin* in both diets showed a significant drop in expression between the initial and P1 time periods and increased until P4 where expression then leveled off during the rest of the experiment (Fig. 2c). Although there were noticeable changes in expression for both diets during the experimental time course a significant increase or decrease was not found when evaluating over the entire experiment. *FBP* expression decreased from its initial concentration and then leveled out over the course of the experiment without detection of any significant changes in expression between the diets (Fig. 2d).

3.3. Expression of degradation genes

To determine if age/size, affected degradation pathways in rainbow trout, the expression of four degradation related genes was analyzed in liver and muscle tissues isolated from fish reared on both diets. These genes are involved in the caspase, calpain, and proteasomal pathways. The degradation genes analyzed for expression include *proteasome 20 delta (prot20D), caspase-3, calpain1, and calpastatinL* which were evaluated for their expression in both muscle and liver tissue. In the muscle *prot20D* expression showed no difference over time for either diet, fishmeal diet $R^2=0.7$ and plant based diet $R^2=0.61$, $P<0.5$ (Fig. 3a). Expression of *prot20D* in the liver showed no significant differences between fish on the two diets or over the course of the experiment (Fig. 3b). *Caspase-3* expression in muscle showed a significant drop in expression for both diets from the initial time period to P1 (Fig. 3c). Expression then leveled out and remained at relatively constant levels over the remainder of the experiment. In liver this effect was just the opposite, with expression significantly increasing between the initial and P1 time periods (Fig. 3d). However any changes between the diets and over time were insignificant for both diets and between muscle and liver. *Calpain1* expression was mostly linear for both diets and in both tissues over the course of the experiment with only slight significant differences between the diets but no significant changes over time (Fig. 3e and f). For the expression of *calpastatinL*, significant changes were detected in the muscle between the diets at time periods P2 and P6 (Fig. 3g). Fish on the plant based diet showed a significant increase over the course of the experiment, $R^2=0.716$, $P<0.045$. In fish reared on the fishmeal diet no significant changes were found. In the liver, significant changes in *calpastatinL* expression were found between the two diets at time periods P2, P5 and P6 (Fig. 3h). Furthermore, *calpastatinL* expression was also found to significantly decrease over time in fish reared on the fishmeal diet during the experiment, $R^2=0.70$, $P<0.02$. In fish reared on the plant based diet, expression was found to significantly decrease from initial values at time points P2 and P3 and then increase during the last three time periods.

3.4. Enzymatic activity of degradation pathways

Protein isolated from the muscle and liver of sampled fish was analyzed for the enzymatic activity of three degradative pathways. Analysis of activity for the ubiquitin-proteasome pathway in muscle showed a significant decrease over the course of the experiment for both diets, fish meal diet $R^2=0.734$, $P<0.007$ and plant based diet 2 $R^2=0.77$, $P<0.002$ (Fig. 4a). The levels of activity were similar between the two diets with exception of a significant difference found at time period P5. In the liver significant differences in activity were found between the diets during analyses at several of the sample time points, specifically at P1, P3, and P6 (Fig. 4b). Fish reared on the fishmeal diet also demonstrated a significant increase in activity over the course of the experiment, $R^2=0.86$, $P<0.001$ while activity for the plant meal diet had an $R^2=0.03$, $P<0.34$. Caspase-3 activity levels in

muscle were similar with only time period P2 showing any significant differences (Fig. 4c). Activity for both diets tended to decrease over the time course of the experiment, however not significantly (4d). Calpain1 enzymatic activity significantly declined in muscle over the course of the experiment in fish on both diets, fish meal diet R^2 0.98 and $P<0.001$, plant-based diet $R^2=0.84$ and $P<0.002$ and there was no significant difference in activity level between the diets at any time point (Fig. 4e). Calpain activity in the liver was completely opposite with activity increasing significantly in fish on both diets, fish meal diet $R^2=0.88$ and $P<0.001$, plant-based diet $R^2=0.78$ and $P<0.001$, but again there were no significant changes in activity between the diets at any of the measured time points (Fig. 4f).

4. Discussion

Animal growth relies on a number of different cellular systems acting in concert to utilize available energy for muscle growth. However, muscle growth not only depends upon the synthesis of incoming protein but also relies upon protein degradation in existing muscle. The interactions between different cellular and organ systems that are responsible for controlling protein turnover are unknown. In fish, research has shown that changes in dietary intake can have pronounced effects on metabolism (Houlihan et al., 1988; Peragon et al., 1999; Kolditz et al., 2008). Use of unrefined plant material has been shown to be immunoreactive in some instances and recent findings have demonstrated that genetic variation exists between rainbow trout families and their ability to grow on a plant protein based diet as compared to fishmeal formulated feed (Pierce et al., 2008). Studies utilizing formulated diets that contain plant proteins have found significant changes for the expression of genes involved in multiple pathways and the level of proteins related to metabolic pathways and protein turnover (Martin et al., 2003; Kolditz et al., 2008). In this experiment the expression of muscle growth related factors and the activity of enzymes responsible for protein degradation were studied during the early active growth stages of rainbow trout and compared between fish that were fed either a fishmeal or plant protein based diet.

4.1. Expression of muscle growth related factors

The gene expressions evaluated in this experiment in muscle have been examined in other fish studies and changes determined according to the nutritional state of the animal (Panserat et al., 2001; Johansen and Overturf, 2006). For most of the genes tested there was not a significant difference in expression level found between the two diets, specifically for the genes *Tmyostatin1, Tmyogenin,* and *fructose 1,6-bisphosphatase* over the course of the experiment. However, significant changes were found in the expression of *TMyoD2* between the diets for time period P2 through P5 (Fig. 2a). Because the action of MyoD in muscle development involves terminal differentiation of muscle cells, the increased level in the muscle of the smaller fish being fed the plant protein formulated diet is not something that would normally be predicted. In previous studies evaluating fish fed a formulated fish meal feed, expression changes of *TMyoD2* at different growth phases were similar to the findings in this study (Johansen and Overturf, 2005). However, in the fish fed a plant-based diet there is a significant increase in expression over that of the fish fed the fishmeal diet. This increase in expression peaks at time period P2 (~16 g) and then decreases until time P6 (~400 g) where its expression level becomes similar to that seen in fish reared on the fishmeal based diet. *MyoD* is a key gene for the regulation of muscle differentiation and is believed to be involved with hyperplastic muscle growth in fish (Rescan, 2005). But even though the expression of *TMyoD2* is higher in these fish on the plant protein based diet the actual weight of these fish is less than that for the fish fed the fishmeal diet at these same early time periods. From the experimental analysis of this study it is not possible to precisely determine how the components of the plant-based diet are modulating the expression of MyoD2 in these fish. However, there is research regarding MyoD regulation that might explain some of the changes seen in this study.

The differences in MyoD2 expression demonstrate an inverse correlation with what is found in regards to the weight of the fish on the two diets over the course of the experiment. Usually increased *TMyoD2* expression equates with increased muscle development and growth but it might be possible that metabolic processing of the plant-based protein diet might not be as efficient in these fish due to reduced glucose receptor expression. Vinals et al. (1997) found that an increase in MyoD protein decreased expression of the glucose receptor GLUT1 while simultaneously upregulating expression of GLUT4. This could affect glucose uptake and available energy. Also TNFα is known to destabilize MyoD proteins and limit muscle development (Langdon et al., 2004). Other researchers have shown than an increase in *TNFα* expression occurs when fish are fed diets containing soybean meal (>20%) or from chronic immune stimulation (Johansen et al., 2006; Sealey et al., 2008). This could potentially explain why these fish weigh less even though they are expressing MyoD at a higher level. However, during the destabilization of MyoD by TNFα the destabilized protein is targeted by ubiquination and degraded in the proteasome in mammals. Increased proteasome activity is not detected in the muscle of the fish on the plant protein based diet during this time frame but regulation of protein degradation, specifically in fish muscle, has been reported to be different than in mammals (Dobly et al., 2004). Furthermore, MyoD is expressed in activated satellite cells, its activation prevents the proliferation of these cells by upregulating the expression of the cell cycle inhibitor p21 (Berkes and Tapscott, 2006). It is possible that MyoD is being continually expressed in these cells preventing their commitment to myoblasts, and limiting muscle development and growth. Regulation at this point without further commitment could explain the enhanced MyoD levels and the growth differences seen between fish on the two diets.

Expression of *Tmyostatin1* is not significantly different between fish on the two diets and follows a pattern that has been previously reported with an increase in expression early in development followed by a reduction and then a gradual increase in growing fish after 100 g (Johansen and Overturf, 2005). Although Tmyostatin2 has been postulated to be involved with metabolic regulation, the action of Tmyostatin1 has generally been proven to function similar to the mammalian isolated myostatin gene and functions to restrict the expression of muscle specific factors related to proliferation and differentiation (Rescan, 2005).

Tmyogenin initially shows a significant drop in expression and then gradually increases until time period 3 before leveling off and finally decreasing between time periods P5 and P6. The relative change of Tmyogenin was insignificant over the course of the experiment and its relative change was similar to what has been witnessed prior in measurements in similar sized fish (Johansen and Overturf, 2005). For the expression of fructose 1,6-bisphosphatase there is also a decrease in expression between the initial time period and P1, then expression level and is constant over the remainder of the experiment. In prior experiments the expression of this gene had been found to vary in muscle during starvation and refeeding, but with constant growth its expression appears to be relatively constant (Johansen and Overturf, 2006).

4.2. Expression of degradation genes and enzymatic activity of degradation factors during early rainbow trout growth

The expression and enzymatic activity of degradation factors was evaluated in both muscle and liver of fish fed the two diets. Muscle accretion relies upon both protein synthesis and degradation and the multiple factors that make up each system. Synthesis depends on growth factors, available energy, and metabolic pathway regulation.

Degradation also relies upon synthesis related components but is accomplished mainly through several identified pathways. From the findings reported here it would appear that all the protein degradation pathways are active to some extent in fish muscle. Expression of the delta subunit of proteasome 20 remained relatively stable in the liver of fish on both diets. However, in fish reared on the fishmeal protein diet a significant increase in enzymatic activity was detected in the liver over the course of the experiment with higher activities seen at time periods P1 and P3, and significant decreased levels seen at P6, as compared to expression from the livers of fish reared on the plant based diet. Yet even though activity in the liver of fish on the fishmeal based diet rose significantly and no significant increase or decrease was seen for fish on the plant based diet over the course of the experiment the average level of activity between fish on the two diets was not significantly different.

In the muscle, expression of proteasome 20 was unchanged during fish growth. However, proteasome enzymatic activity decreased significantly in the muscle of fish reared on both diets as fish size increased. Other research suggests fish with lower growth efficiencies have increased protein synthesis rates and higher degradation rates (Dobly et al., 2004). Extrapolating from their findings it would appear, in regards to proteasome activity in the muscle, there is no significant difference in the utilization of plant proteins instead of fishmeal protein. These findings still suggest that as rainbow trout get bigger muscle proteasome activity decreases, which would benefit muscle accretion and weight gain, and that the fish is becoming more efficient in building muscle as it grows.

Caspase-3 gene expression showed opposing changes in liver and muscle for fish on both diets between the initial samples and time period P1. Yet over the course of the entire experiment the level of caspase-3 remained relatively level, both for expression and for analyzed activity. As apoptosis is not thought to be a primary method for cellular turnover of growing muscle this lack in changing activity is not surprising.

Caspase-3 enzymatic activity appeared to decrease in the liver as the animal grew but the overall change was not found to be significant. In the liver the activity level was relatively constant until time period P3 when activity levels began to climb and then slightly decreased again at the last time period studied, P6. As in muscle the changes seen were not of sufficient amplitude to be determined as significant. In both the muscle and the liver of tested animals there was no significant difference between the fish on either diet, except for P1 in muscle.

A great deal of research regarding calpain activity and its regulation in rainbow trout has been reported by Salem et al. (2004, 2005a,b, 2006a,b). From these findings and reported activities of calpain in the degradation of muscle components in mammals it was thought that this might be a major pathway for regulating muscle turnover (Huang and Forsberg, 1998). Though there were some significant differences at time periods P3 and P4 in muscle and liver between fish on the separate diets, the relative level of expressed *calpain1* was similar over the course of the experiment in both tissues. Our findings for *calpastatin* L expression however showed significant differences between the diets in both liver and muscle. In the muscle, *calpastatin* L expression was stable for fish on the fish meal formulated diet but showed a significant increase in expression over the course of the experiment in fish fed the plant-based feed. In the liver, fish fed the plant-based feed showed differences in expression of *calpastatin* L during the time periods of P2, P4 and P5, compared to the fishmeal based diet, the level then increased at P6 but when evaluated over the course of the entire experiment the overall change was found to be nonsignificant. However in the liver of fish fed the fishmeal diet there was a significant decrease in expression of *calpastatin* L over the course of the experiment.

In the muscle of fish on both diets, calpain activity showed a significant decrease of 25% in activity over the time course of the experiment. Like with proteasome activity this finding demonstrates a reduction in muscle degradation as the animals become bigger. In the liver though, calpain activity increased 33% between the initial time period and P6. Research in this area has been confounding, with some studies showing a decrease in activity for some degradation pathways over time and others showing an increase in activity over time (Hayashi and Goto, 1998). But most studies have been done in animals that have determinate growth levels while only a limited number of studies in fish have looked at degradation activity in tissues over time (Buttery et al., 1975; Guerin, 2004).

Calpastatin is a negative regulator of calpain activity and these finding show a correlation in muscle for fish on the plant based diet, but not the fishmeal diet in muscle. Conversely in the liver the decrease of *calpastatin* L expression for fish fed the fishmeal diet inversely correlates with the detected increase in calpain activity. Although the fish fed the plant based diet also showed an increase in calpain activity in the liver, the expression of *calpastatin L* does not demonstrate the inverse correlation that is seen for the fish on the fishmeal diet. Interestingly, calpastatin, like MyoD, has been shown to be involved in the regulation of glucose transporters and also myocyte enhancing factors (Otani et al., 2004). It is possible that the reason that *calpastatinL* expression levels are not elevated in the muscle of fish on the fishmeal based diet, particularly during the later sampling points, is because these fish are regulating calpain activity by some other means. Research has found that many other chemicals can also effectively reduce or block calpain activity, some of which are also linked to myogenic and glucose regulation (Kook et al., 2008; Tie et al., 2008). The reason for these differences between the diets and tissue is perplexing and points toward a complex interaction of systems for the regulation of nutrient partitioning and protein turnover.

The findings presented here demonstrate how muscle protein degradation rates can differ during the growth of the fish within different degradation systems. Because of the different substrates and methods used to analyze the activity of the different degradation systems determination of the relative contribution of each system to the overall level of degradation, and hence protein turnover, cannot be deciphered. Although some interesting differences were noticed at different time points for the expression of some muscle factors, over the entire time course of the experiment only *TMyoD2* showed differences that varied by diet and changed with fish size. Expression of the *proteasome 20 delta subunit* in the muscle also decreased as fish grew, irrespective of diet which followed the same trend as activity of muscle proteasome 20. In the liver, proteasome 20 activity was altered with fish size on the fish meal based diet but expression was not. Although *calpastatinL* expression and calpain1 activity showed the expected inverse correlation as would be expected in the muscle of fish fed the plant based diet and in the liver of fish fed the fish meal diet. The relative expression and activities of these genes and proteins in these tissues on the opposing diets makes it difficult to determine the physiological interactions that are involved here. Future research will need to address the relative contribution of specific degradation pathways and their individual components and should attempt to determine how different metabolic, immunologic, and other pathways interact with nutrient components to control the rate of muscle synthesis and degradation.

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